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Neutralization of the oedematogenic activity of Bothrops Jararaca venom on the mouse paw by an antibothropic fraction isolated from Opossum (Didelphis Marsupialis) serum

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Abstract

The pharmacological modulation of mice paw oedema produced by *Bothrops jararaca* venom (BJV) has been studied. Intraplantar injection of BJV (1–30 μg/paw) produced a dose- and time-related oedema, which was maximal 30 min after injection, reduced gradually thereafter and disappeared over 48 h. BJV heated at 100°C for 5 or 15 min blocked local hemorrhage and caused partial inhibition of its oedematogenic activity. The BJV oedema was not inhibited by the anti-histamine meclizine, the inhibitor of histamine and serotonin, cyproheptadine, PAF-acether antagonist WEB 2170 or by the anti-leukotrienes C_4/D_4, LY 171883. Dexamethasone, aspirin, indomethacin, and the dual cyclooxygenase and lipoxygenase inhibitor BW 755C inhibited BJV-induced oedema indicating that arachidonic acid metabolism products via the cyclooxygenase pathway participate in its genesis and/or maintenance. The antibothropic fraction (ABF) (25–200 μg/paw) isolated from *Didelphis marsupialis* serum neutralized the oedema induced by the venom with and without heating, the hemorrhage induced by BJV and partially blocked the oedema induced by bradykinin and by cellulose sulphate. The oedema produced by histamine, serotonin, PAF-acether or leukotriene C_4 was not inhibited.

Introduction

Crotalidae snake venoms are known to produce local tissue damage, such as hemorrhage, erythema, oedema and myonecrosis. Besides these local effects, crotalic venoms can produce systemic effects like shock, changes in the coagulation system, platelet aggregation, systemic hemorrhage and liberation of pharmacologically active substances, such as histamine, serotonin and bradykinin [1–4]. Some of the effects induced by these venoms are related to their high proteolytic activity [5–7].

The ability of several snake venoms to increase vascular permeability and to induce oedema has been demonstrated. Such effects were related, at least in part, to the ability of snake venoms to induce liberation of pharmacologically active substances [8–11].

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The oedematogenic activity of *Bothrops jararaca* venom (BJV) was demonstrated by intraplantar injection in rat paw; this process, seems to be partially mediated by BJV proteolytic activity and is largely dependent on both cyclooxygenase and lipoxygenase products of arachidonic acid metabolism [12].

The resistance of some animals to certain snake venoms has been demonstrated. In many cases, this resistance could be explained by the presence of neutralizing factors in their blood sera ([13–19], for review see reference [20]).

The ability of the opossum (*Didelphis marsupialis*) serum to inhibit the lethal action of *B. jararaca* venom has already been described [16, 21]. A proteinaceous complex with anti-*Bothrops jararaca* venom activity was isolated from the same serum [22, 23]. This antibothropic factor (ABF) protected mice against the lethal effect of *B. jararaca* venom, and rabbit against hemorrhage and myonecrosis induced by the same venom [24].

The purpose of this study was to evaluate pharmacologically the mouse paw oedema induced by BJV and to investigate the ability of the antibothropic fraction, isolated from *Didelphis marsupialis* to neutralize this oedema.

Material and methods

Antibothropic fraction (ABF) isolation

ABF was prepared according to a previously described method [23]. Briefly, *Didelphis marsupialis* serum was dialyzed for 24 h at 4°C against 0.01 *M*, pH 3.7 sodium acetate. After centrifugation the supernatant was directly fractionated on a DEAE-Sephacel column (Pharmacia K 26/40) and eluted, initially with 0.01 *M* acetate buffer and finally with the same buffer containing 0.15 *M* sodium chloride. The active fraction was pooled, dialyzed against 0.01 *M* ammonium carbonate, freeze-dried and stored at 4°C until use.

Production of paw oedema by BJV and other drugs

Male Swiss-webster mice weighing 25–30 g were used. The paw oedema was induced by intraplantar injection of BJV (1, 3, 10 and 30 μg/paw), histamine (50 μg/paw), serotonin (100 μg/paw), bradykinin (50 μg/paw), PAF-acether (1 μg/paw), leukotriene C_4 (1 μg/paw) or cellulose sulphate (300 μg/paw), dissolved in 50 μl of sterile 0.9% NaCl (saline), in one of the mice hind footpads. The same volume of sterile saline was injected into the contralateral paw. The oedema was measured by plethysmography [25] at different times after injection of BJV (0.5, 1, 3, 4, 6, 24, 48 and 72 h), 0.5, 1 and 3 h after injection of cellulose sulphate or 0.5 h after injection of the other drugs. Results were expressed as the difference between the values obtained after injection of BJV or drugs and the saline control. BJV heated at 100°C in a water bath for 5 and 15 min was also used to induce mice paw oedema for testing the influence of heating on the oedematogenic ability of the BJV.

Drugs treatments

Different groups of animals were treated intraperitoneally (i.p.) 1 h before injection of BJV (10 μg/paw), with the following drugs: the H_1 antagonist meclizine (7.5, 15, 30 mg/kg), the dual cyclooxygenase and lipoxygenase inhibitor BW 755C (5, 10, 20 mg/kg), the cyclooxygenase inhibitors aspirin (50, 100, 200 mg/kg) and indomethacin (0.5, 1, 2 mg/kg), the dual histamine and serotonin inhibitor cyproheptadine (0.5, 1, 2 mg/kg), the corticosteroid dexamethasone (0.1, 0.3, 0.5 mg/kg), the PAF antagonist WEB 2170 (4, 8, 16 mg/kg) and the leukotriene C_4/D_4 inhibitor LY 171883 (30 mg/kg). All drugs were dissolved in sterile saline solution just before use.

Antibothropic fraction treatments

ABF, previously dissolved in saline (25, 50, 100 or 200 μg/paw) was administered together with the following oedematogenic agents: BJV with and without heating (10 μg/paw), histamine (50 μg/paw), serotonin (100 μg/paw), bradykinin (50 μg/paw), leukotriene C_4 (1 μg/paw), PAF-acether (1 μg/paw) and cellulose sulphate (300 μg/paw). The possible anti-oedematogenic activity of ABF was determined at different time intervals listed under Results section following the same plethysmography procedure [25].

Venom and Drugs

Bothrops jararaca venom was kindly supplied by Dr. C. R. Diniz from Ezequiel Dias Institute, Belo Horizonte, MG, Brazil. LY 171883 (1-[2-hydroxy-

3-propyl-4-[4-(1H-tetrazol-5-yl)butoxy] phenyl] ethanone) was obtained from Eli Lilly and Company (Indianapolis, USA); WEB 2170 (6-(2-chlorophenyl)-8,9-dihydro-1-methyl-8-(4-morpholinylcarbonyl)-4H, 7H-cyclo-pental (4,5) thieno (3,2-f) (1,2,4)-triazolo-(4,3-a) (1,4)-diazepine was supplied by Boehringer-Ingelheim (FRG); BW 755C (3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride) by Wellcome Research Laboratories; PAF-acether (1-0-hexadecyl-2-acetyl-sn-glyceryl-3-phosphoryl-cholyne was purchased from Bachem (Switzerland); aspirin (Aspegic-lysine salt) from Laboratoires Egic (France); dexamethasone (Decadron) from Merck Sharp & Dohme (Brazil); leukotriene C_4, indomethacin, cyproheptadine, histamine, serotonin and bradykinin were purchased from Sigma (USA).

Statistical analysis

The data were statistically microcomputer-treated using an analysis of variance program (ANOVA) followed by Newman Keuls Student's *t*-test. *p* values of 0.05 or less were considered significant.

Results

Bothrops jararaca venom oedematogenic activity

The intraplantar injection of BJV in doses between 1 and 30 µg/paw into one of the hind paws of mice displayed a dose- and time-related paw oedema, which was maximal 30 min after injection (Fig. 1). All venom doses induced hemorrhage together with the oedema (data not shown). BJV previously heated at 100 C for 5 or 15 min produced a non-hemorrhagical oedema; a statistically significant reduction of this oedema was observed 4 h after injection (Fig. 2).

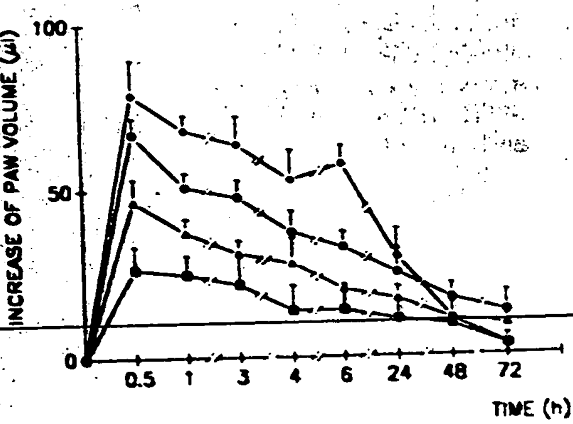

Figure 1
Dose- response curve and kinetics of BJV-induced paw oedema. BJV was injected in mice by intraplantar route, using 1 (■), 3 (▲), 10 (●) and 30 (●) µg/paw. Each point is the mean from at least six animals. Vertical lines represent SEM.

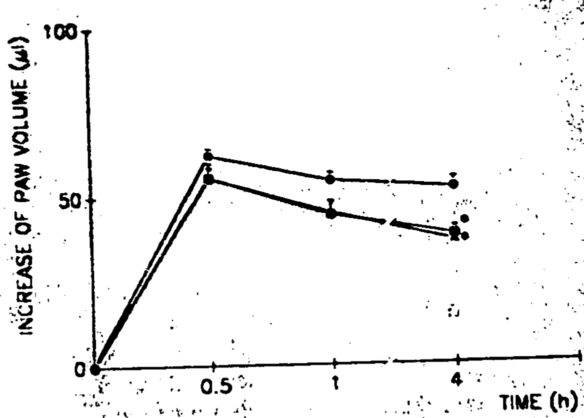

Figure 2
Effect of heating of *Bothrops jararaca* venom on its oedematogenic activity. BJV (10 µg/paw) (●); BJV (10 µg/paw) heated at 100 C for 5 min (▲) and BJV heated at 100 C for 15 min (■) were intraplantarly injected in mice and the oedema was measured at different time intervals. Each point is the mean from at least six animals. Vertical lines represent SEM. Statistically significant differences are indicated by $^{*}p < 0.05$.

Pharmacological characterization of BJV-induced oedema

The i.p. pretreatment of mice with the H_1 antagonist meclizine, the inhibitor of serotonin and histamine cyproheptadine, the PAF antagonist WEB 2170 and the leukotriene C_4/D_4 inhibitor LY 171883 did not reduce the oedema produced by BJV at none of the times or doses used (Fig. 3). However, when drugs which interfered with the arachidonate metabolism, such as the cyclooxygenase inhibitors aspirin and indomethacin, the dual cyclooxygenase and lipoxygenase inhibitor BW 755C and the corticosteroid dexamethasone were used, it was observed that all of them caused significant inhibition of the oedematogenic activity of BJV (10 µg/paw) (Fig. 4), but did not inhibit the hemorrhage induced by the same venom (data not shown).

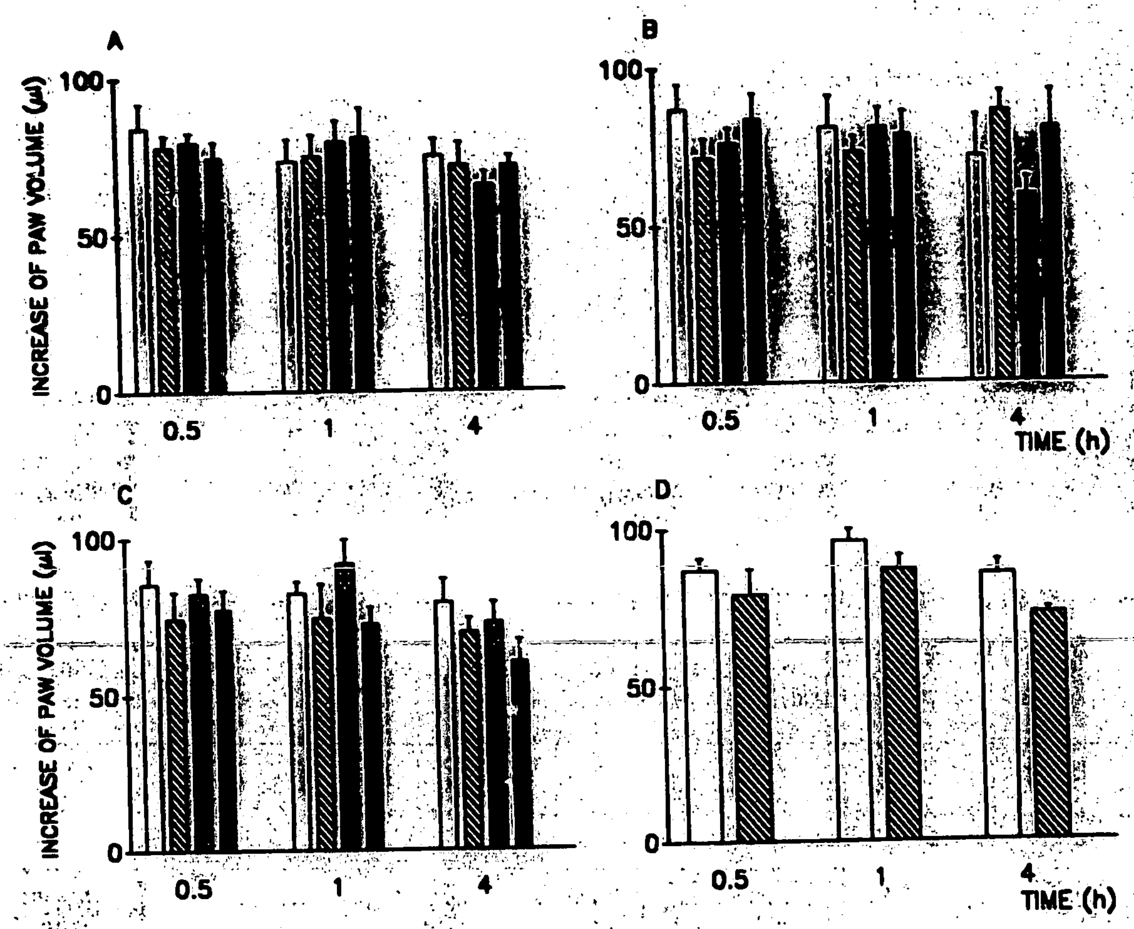

Figure 3
Effect of i.p. treatments of (A) meclizine, (B) cyproheptadine, (C) WEB 2170 and (D) LY 171883 on *Bothrops-jararaca*-induced paw oedema. All drugs were i.p. injected in mice 1 h before BJV injection (10 μg/paw). (A) Saline (□), meclizine 7.5 (▧), 15 (▩) and 30 (■) mg/kg. (B) Saline (□), cyproheptadine 0.5 (▧), 1 (▩) and 2 (■) mg/kg. (C) Saline (□), WEB 2170 4 (▧), 8 (▩) and 16 (■) mg/kg. (D) Saline (□), LY 171883 30 mg/kg (▧). Each column represents the mean from at least six animals with SEM indicated by vertical lines.

Aspirin caused inhibition of the BJV-induced oedema at all times measured, when the 200 mg/kg dose was used (35% at 30 min, 37% at 1 h and 50% at 4 h); lower doses of aspirin (50 and 100 mg/kg) also inhibited this oedema (32% and 35% respectively), but only 4 h after BJV injection (Fig. 4A). Indomethacin (Fig. 4B) and dexamethasone (Fig. 4D) inhibited both 1 and 4 h BJV-induced paw oedema at all doses used, but did not inhibit it at 30 min. The highest inhibition produced by dexamethasone (0.5 mg/kg) and indomethacin (2 mg/kg) was 43% and 42%, 4 h after BJV injection. BW 755C reduced the 1 and 4 h oedema produced by BJV at all doses used (5, 10 and 20 mg/kg) (Fig. 4C); the highest dose produced the highest inhibition (40%). None of the doses used inhibited this oedema 30 min after BJV injection.

Anti-oedematogenic activity of ABF

The ability of ABF to inhibit the oedematogenic activity of BJV is shown in Fig. 5. The concomitant injection of ABF (25, 50, 100 and 200 μg/paw) with BJV (10 μg/paw), inhibited the BJV-induced oedema. Doses of 100 and 200 μg/paw inhibited the

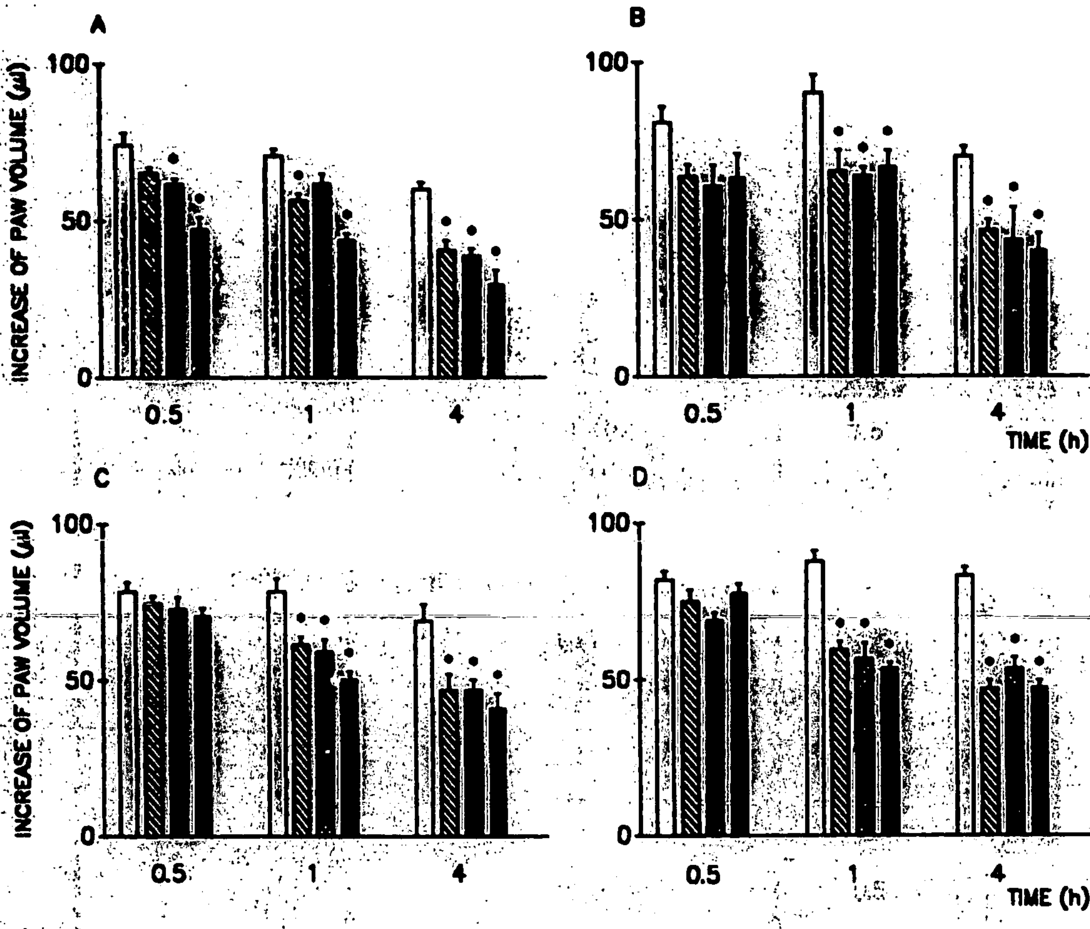

Figure 4
Effect of i.p. treatments of (A) aspirin, (B) indomethacin (C) BW 755C, and (D) dexamethasone on *Bothrops-jararaca*-induced paw oedema. All drugs were i.p. injected in mice 1 h before intraplantar injection of BJV (10 µg/paw). (A) Saline (□), aspirin 50 (▨), 100 (▩) and 200 (■) mg/kg. (B) Saline (□), indomethacin 0.5 (▨), 1 (▩) and 2 (■) mg/kg. (C) Saline (□), BW 755C 5 (▨), 10 (▩) and 20 (■) mg/kg. D saline (□), dexamethasone 0.1 (▨), 0.3 (▩) and 0.5 (■) mg/kg. Each column represents the mean from at least six animals with SEM indicated by vertical lines. Statistically significant differences are indicated by $^{*}p < 0.05$.

30 min oedema by 26.6% and 32%, respectively; all doses of ABF used inhibited the 1 and 4 h BJV-induced oedema; the highest inhibition observed was of 60%, obtained with 100 µg/paw and 4 h after injection of venom plus ABF. The ABF also inhibited the hemorrhage induced by BJV (results not shown).

When ABF (200 µg/paw) was injected with previously heated BJV (10 µg/paw), it also inhibited the oedematogenic effect of this venom (Fig. 6). ABF inhibited the 5 min preheated BJV-induced oedema at the three measured times, 0.5, 1 and 4 h, by 27%, 40% and 46%, respectively, and also inhibited the 15 min preheated BJV-produced oedema at the same times by 32%, 48% and 45%. ABF (100 and 200 µg/paw) also inhibited the oedema induced by bradykinin (50 µg/paw) (Fig. 7A) and cellulose sulphate (300 µg/paw) (Fig. 7B). ABF was not able to reduce the oedema induced by either histamine, serotonin, PAF-acether, or leukotriene C_4 at none of the doses used (Fig. 8).

Discussion

The oedematogenic activity of some snake venoms or their fractions has been demonstrated previously [11, 26–29]. However, in the case of BJV, only

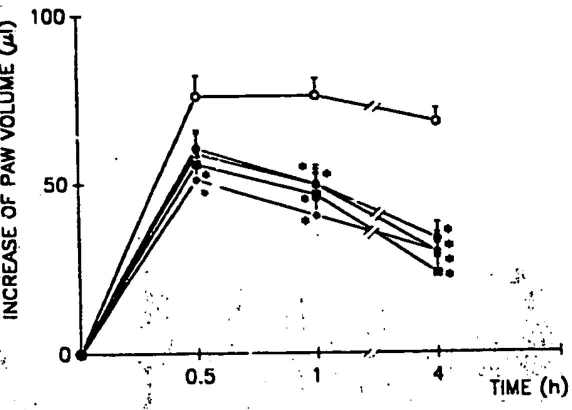

Figure 5
Effect of the antibothropic fraction (ABF) isolated from *Didelphis marsupialis* serum on *Bothrops-jararaca*-induced paw oedema. BJV (10 μg paw) was intraplantarly injected in mice, alone (○) or together with ABF 25 (●), 50 (▲), 100 (■) and 200 (◆) μg paw. Each point represents the mean from at least six animals with SEM indicated by vertical lines. Statistically significant differences are indicated by * $p < 0.05$.

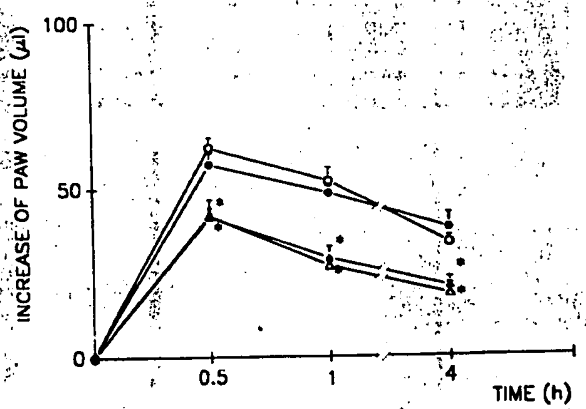

Figure 6
Effect of the antibothropic fraction (ABF) isolated from *Didelphis marsupialis* serum on preheated *Bothrops jararaca*-induced paw oedema. BJV (10 μg paw) heated at 100 C for 5 min (●) or 15 min ( ) was intraplantarly injected in mice, alone or together with ABF 200 μg paw (▲) and (△) respectively. Each point represents the mean from at least six animals with SEM indicated by vertical lines. Statistically significant differences are indicated by * $p < 0.05$.

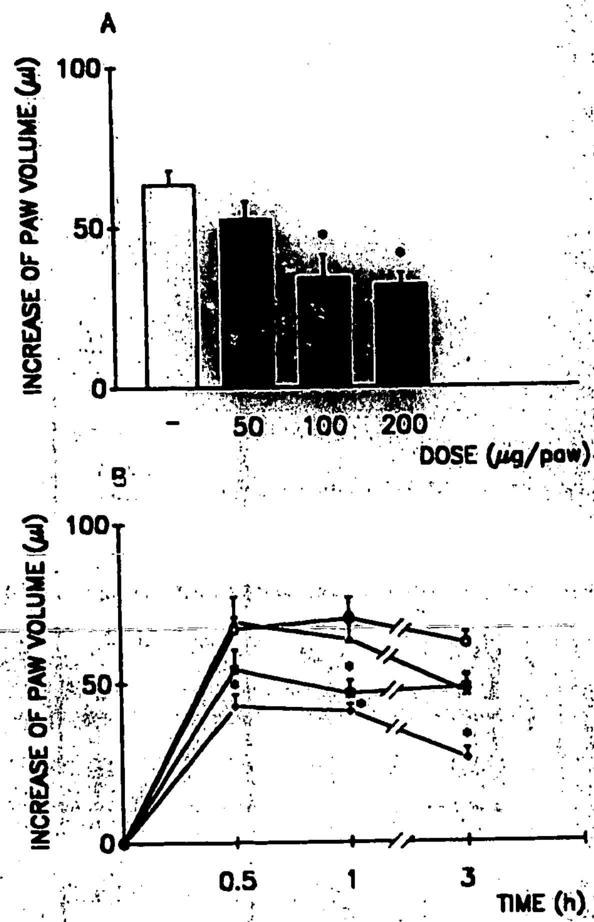

Figure 7
Effect of the antibothropic fraction (ABF) isolated from *Didelphis marsupialis* serum on the oedema induced by (A) bradykinin and (B) cellulose sulphate. (A) Bradykinin (50 μg/paw) was intraplantarly injected in mice, alone (white column), or with ABF, and the oedema was measured 30 min after that. (B) Cellulose sulphate (300 μg/paw) was intraplantarly injected alone (○) or with ABF 50 (▲), 100 (■) and 200 (◆) μg/paw. Each point or column represents the mean from at least six animals with SEM indicated by vertical lines. Statistically significant differences are indicated by * $p < 0.05$.

recently has it been studied systematically. Trebien and Calixto (1989) [12] showed the ability of BJV to produce rat paw oedema. In the present investigation, we demonstrated the ability of this venom to induce oedema in mice paw, in a dose- and time-related process. This oedema was maximal 30 min after venom injection and disappeared totally only over 48 h. This result is slightly different from that obtained by Trebien and Calixto [12] using a similar venom in rats; in their case the oedema was maximal within 1 h and disappeared within 24 h after venom injection. Studies using other venoms have showed that the kinetics of oedema induced

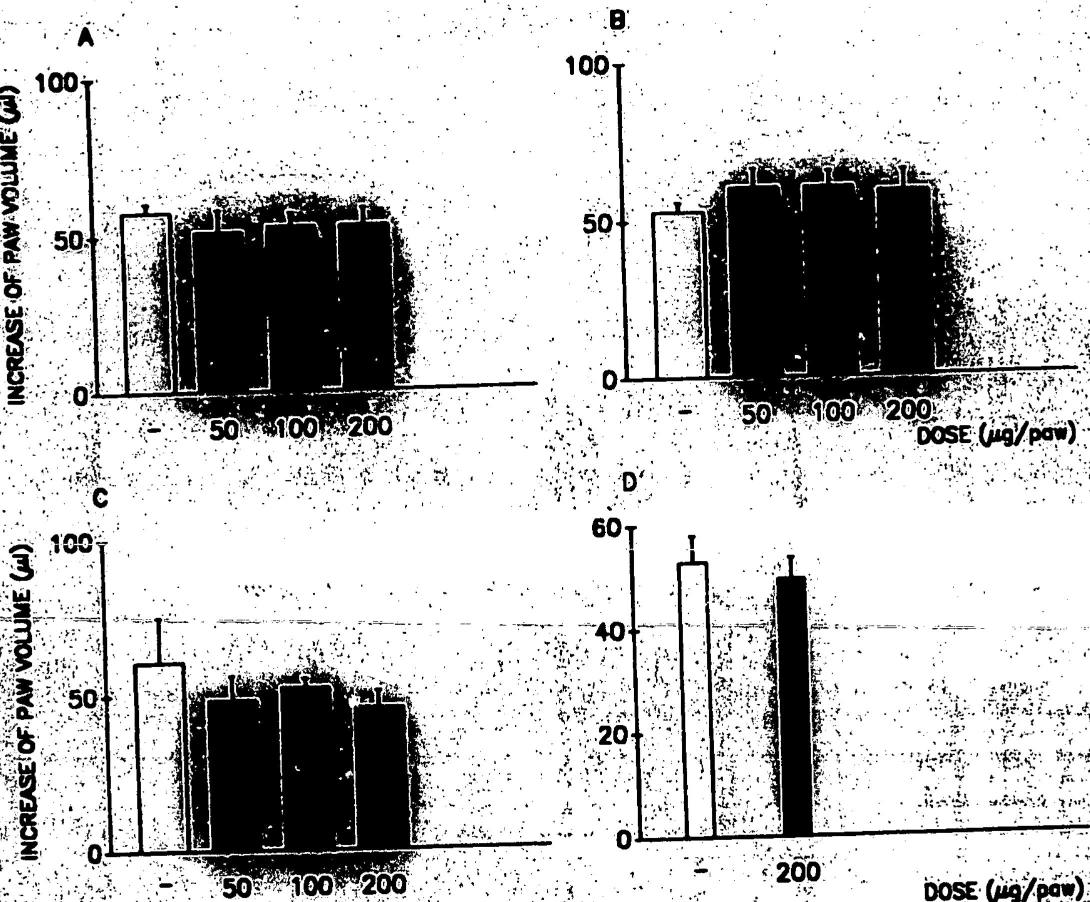

Figure 8
Effect of the antibothropic fraction (ABF) isolated from *Didelphis marsupialis* serum on oedema induced by (A) histamine (B) serotonin. (C) PAF-acether and (D) C_4 leukotriene. Histamine (50 μg/paw), serotonin (100 μg/paw), PAF (1 μg/paw) and C_4 leukotriene (1 μg/paw) were intraplantarly injected in mice, alone (□) or together with ABF (■) and the oedema was measured 30 min after that. Each column represents the mean from at least six animals with SEM indicated by vertical lines.

by different snake venoms is also different. In all cases, however, it was reported that the beginning of the process was too fast, and that the time to reach the maximal effect was very different [11, 26, 28, 29].

The participation of hemorrhagic factors in the oedematogenic activity of BJV was showed. It was observed, however, that this participation was only partial, because when the hemorrhagic activity was completely blocked by heating the venom at 100°C for 5 min, the oedema was reduced to a maximum value of only 27%, 4 h after BJV injection. When the same venom was heated at the same temperature for 15 min, no additional reduction of the oedema was observed. This would indicate that the oedematogenic activity of BJV depends on the heat-labile proteolytic enzymes, like the hemorrhagic factors [30, 31], but is independent of the heat-stable proteolytic enzymes, like Bothrops protease A, thrombin-like enzymes or kininogenases [32, 33]. The oedematogenic property of some hemorrhagic factors isolated from different snake venoms has been previously demonstrated [27, 29]. For instance, Queiroz et al. [27] showed the oedematogenic activity of HF_2, an hemorrhagic factor isolated from BJV.

The study of the participation of several inflammatory mediators on BJV-induced mice paw oedema

showed that, the H_1 antagonist meclizine, the dual inhibitor of serotonin and histamine cypr heptadine, and the PAF-acether antagonist WEB 2170 did not inhibit the oedema induced by BJV, suggesting the non-participation of histamine, serotonin and PAF-acether in the pathogenesis of this oedema. On the other hand, when drugs which interfered with the metabolism of the arachidonic acid, such as the corticosteroid dexamethasone, the cyclooxygenase inhibitors aspirin and indomethacin, and the dual lipoxygenase and cyclooxygenase inhibitor BW 755C, were used, it was observed that all of them caused significant inhibition of the BJV oedematogenic activity. However, when the leukotriene C_4/D_4 inhibitor LY 171883 was used, it did not reduce the oedema triggered by BJV at none of the times or doses used. These results suggest the participation of arachidonate metabolites, obtained via the cyclooxygenase pathway, in the pathogenesis of BJV-induced mice paw oedema, and the non-participation of leukotrienes C_4/D_4 in the genesis and/or maintenance of BJV oedema. Our results do not discount the possible participation in the mouse paw oedema of arachidonate metabolites produced via the lipoxygenase pathway different from peptidyl leukotrienes (such as LTB4). On the other hand, the effect of dexamethasone should be interpreted not only on the basis of its interference with arachidonic acid metabolism but also with the involvement of some alternative mechanism (like the inhibition of leukocyte migration or the inhibition of lymphokine synthesis). The participation of different metabolic products of the arachidonic acid in the development of oedema induced by some snake venoms, had been suggested by others [12, 28, 34]. Trebien and Calixto [12], showed the participation of the cyclooxygenase and lipoxygenase eicosanoid products in the development of rat paw oedema caused by the injection of BJV. This result is different from ours, because we did not find any participation of the lipoxygenase derivatives in the genesis of the BJV-induced mice paw oedema. These conflicting resuls could be explained by different BJV origins and/or animal models used in both studies.

The most important local effects of crotalic poisoning are hemorrhage, oedema and myonecrosis. In severe cases of poisoning these effects can lead to complete destruction and posterior loss of the bitten extremity. *Bothrops jararaca* is the most common venomous snake in Brazil and it is responsible for the majority of accidents with snakes in this country; these accidents are also characterized by severe local tissue damage. These local effects produced by crotalidae venoms are not effectively neutralized by antivenoms when mice or rabbits are used [3, 35–37].

The oedematogenic activity of some snake venoms is not effectively blocked by other agents like the detoxified serum of the non-venomous snake *Clelia clelia*, which inhibits the lethal action of some snake venoms [38], the antihemorrhagic alkaloid aristolochic acid [28] and the crude extract of *Mandevilla velutina*, a plant used in Brazil for the treatment of snake bites [12].

In this work we demonstrate the ability of ABF to inhibit the oedematogenic activity of BJV. ABF significantly inhibited the BJV mice paw oedema induced by the venom with and without heating. It is shown that ABF blocked not only the oedema induced by the hemorrhagic factors present in the venom, but also the oedema produced by other venom fractions. Previously, we showed the ability of ABF to inhibit the hemorrhagic effect produced by BJV and by a hemorrhagic fraction isolated from the same venom [24, 39]. Neutralization of the hemorrhagic activity of several other snake venoms by the opossum and wooodrat sera has been shown by others [40].

ABF produced significant inhibition of mouse paw oedema induced by bradykinin and by cellulose sulphate. These data seem to indicate the participation of kinins in the mechanism of production of oedema by BJV. However, the oedema produced by histamine, serotonin, PAF-acether or leukotriene C_4 was not inhibited by ABF. These results agree with our data since in the pharmacological characterization of the oedema induced by BJV, it is shown that histamine, serotonin, PAF-acether and leukotriene C_4/D_4 do not participate in the pathogenesis of this oedema.

Since ABF inhibits not only the oedematogenic activity of BJV but also the hemorrhagic, myonecrotic and lethal effects of this venom, this suggests the possible use of this fraction as a helpful therapeutic agent for the treatment of *Bothrops jararaca* and other Crotalidae bites.

In conclusion, in this work we have described the ability of *Bothrops jararaca* venom to induce mice paw oedema in a dose- and time-related process, mediated mainly by cyclooxygenase arachidonic acid metabolism products. Histamine, serotonin, PAF-acether and leukotrienes D_4 and C_4 do not appear to participate in the genesis of this oedema.

We have also sh wn that the antibothropic fracti n isolated from *D. marsupialis* serum, besides inhibiting the oedematogenic activity f *B. jararaca* venom, also inhibits the oedema induced by bradykinin and cellulose sulphate, but not the one induced by hist imine, serotonin, PAF-acether or leukotriene C_4 oedema.

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